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ORIGINAL ARTICLE

Novel markers of endothelial dysfunction and inflammation in Behçet's disease patients with ocular involvement: epicardial fat thickness, carotid intima media thickness, serum ADMA level, and neutrophil-to-lymphocyte ratio

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Abstract The etiology of Behçet's disease (BD) has not been fully elucidated. However, immunological and environmental factors, endothelial dysfunction (ED), and genetic susceptibility have been proposed to play a role. In this study, we aimed to evaluate epicardial fat thickness (EFT) together with serum asymmetric dimethylarginine (ADMA), carotid intima media thickness (CIMT), and neutrophil-to-lymphocyte ratio (NLR) in BD patients with ocular involvement. Thirty-six ocular BD patients (17 active and 19 inactive ocular involvement), and 35 age and sex-matched healthy controls were enrolled to this cross-sectional study. All patients underwent examinations with transthoracic echocardiography and carotid Doppler ultrasound. Serum ADMA levels, CIMT, EFT, and NLR were compared between groups, and their association with disease activity was evaluated. Behçet's disease patients had higher WBC counts, neutrophil counts, NLR, CIMT, EFT values, and serum ADMA levels than do healthy controls. The other biochemical, hematological, and echocardiographic parameters were comparable between the two groups. Behçet's disease duration was positively correlated with EFT and CIMT. Multivariate logistic regression analysis revealed that increased serum ADMA concentration and CIMT are independently associated with BD. Neutrophil counts, NLR, and serum ADMA level were higher, and lymphocyte count was lower in patients with active ocular BD compared to those of inactive ocular BD group. Carotid intima media thickness, serum ADMA level, EFT, and NLR were increased in ocular BD patients compared to healthy subjects. In addition, both serum ADMA level and NLR were associated with disease activity of ocular involvement. Increase in disease duration was associated with increase in CIMT and EFT which suggests that anatomical changes occur in time during the disease course. Increased CIMT, serum ADMA level, EFT, and NLR may provide new clues about the role of ED and inflammation in the etiopathogenesis of BD.

Keywords ADMA · Asymmetric dimethylarginine · Behçet's disease · Carotid intima media thickness · Neutrophil to lymphocyte ratio · Rheumatic diseases

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Introduction

Behçet's disease (BD) is a multisystem inflammatory disorder that is characterized with recurrent episodes of oral and genital

ulcers, skin lesions, and uveitis [1]. Hulusi Behçet, a Turkish dermatologist, first described BD in 1937 [2]. While the etiology of BD remains unclear, endothelial dysfunction (ED) as well as genetic, environmental, and immunological factors had been proposed in previous studies [3]. Acute systemic inflammation and chronic systemic vasculitis are associated with ED [4] and are considered to play important roles in the initiation and progression of vasculitis in BD [5, 6].

Carotid artery intima media thickness (CIMT) is a noninvasive measurement of structural arterial remodeling that provides anatomical clues regarding peripheral endothelial functions [7]. Additionally, asymmetric dimethylarginine (ADMA) is an endogenous analog of L-arginine that can modulate nitric oxide production and is an endogenous competitive inhibitor of nitric oxide synthase (NOS). Thus, increased serum ADMA level is supposed to be associated with ED [8, 9].

Epicardial adipose tissue is a component of total visceral fat that serves as a source of several endocrine and inflammatory mediators [10]. Epicardial fat has been hypothesized to play a pivotal role in the pathogenesis of various cardiovascular diseases (CVDs) [11, 12] and to be associated with unfavorable cardiovascular and metabolic risk profiles in humans. On the other hand, the neutrophil-to-lymphocyte ratio (NLR) in peripheral blood has been proposed as a novel indicator of systemic inflammation. Its predictive and prognostic value has been shown in several CVDs [13, 14].

In this study, we aimed to evaluate epicardial fat thickness (EFT) together with serum ADMA level, CIMT, and NLR in a homogenous group of BD patients with ocular involvement in addition to assess the association of these variables with disease activity.

Materials and methods

Study population

A total of 36 ocular BD patients (17 with active ocular BD and 19 with inactive ocular BD) without overt peripheral vascular involvement other than retinal vessels were recruited in this cross-sectional study. Thirty-five age and gender-matched healthy individuals who admitted to the ophthalmology outpatient clinic having no apparent health problem other than refractive errors (e.g., myopia, hypermetropia, and astigmatism) formed the control group. Behçet's disease patients were diagnosed according to the established guidelines of the International Study Group [15]. All BD patients had panuveitis without retinal vascular occlusion. Active uveitis was defined as having cells in the anterior chamber or vitreous, having macular edema greater than 250 μm, and retinal vasculitis. All patients were consulted in the rheumatology and dermatology departments, and the medications taken by each patient were noted.

Patients with glaucoma (n=4), diabetes mellitus (n=3), cerebrovascular disease (n=1), CVD (n=2), hypertension (n=5), hypercholesterolemia (n=3), malignancy (n=1), infectious diseases (n=3), liver or renal insufficiency (n=4), alcohol or drug abuse (n=1), obesity (BMI≥30) (n=2), Raynaud's phenomenon (n=1), history of hormone replacement therapy (n=2), and antioxidant therapies (n=1) were excluded. In addition, patients with poor echogeneity (n=2) were not included. Two patients refused to sign the informed consent and did not participate to study. This study was approved by the local Ethics Committee, and all subjects provided informed consent before they participated.

Echocardiogram and EFT measurement

All participants underwent transthoracic echocardiography imaging using an echocardiograph equipped with a broadband transducer (Vivid S6, GE Vingmed Ultrasound, Horten, Norway). Measurements were obtained from long-axis and apical four-chamber views according to standard criteria. The echocardiographic images were recorded into a computerized database (EchoPac). The offline EFT measurements were performed by two cardiologists who were blinded to the patient's clinical data. Three weeks later, a second assessment was performed on 15 randomly selected patients to evaluate the inter- and intra-observer variability of EFT measurements. The interobserver variability was 4.1 %, and the intraobserver variability was 3.2 %. Epicardial fat thickness measurements with echocardiography were conducted according to the method previously described by Iacobellis et al. [16]. The epicardial fat appears as an echo-free space between the pericardial layers via 2-D echocardiography. The maximum EFT was measured at the anatomic landmark at the end-diastole of three cardiac cycles, which was located on the free wall of the right ventricle along the midline of the ultrasound beam, perpendicular to the aortic annulus (Fig. 1).

Measurement of CIMT

The carotid intima media thickness was measured by high-resolution B-mode US by a single experienced examiner who was blinded to the clinical and biochemical data. All patients were evaluated with an ultrasound system (Vivid S6, GE Vingmed Ultrasound, Horten, Norway) equipped with a 12-MHz linear array transducer. Carotid intima media thickness was defined as the distance from the leading edge of the lumen-intima interface to the media-adventitia interface of the carotid artery. The intima media thicknesses of the left and right common carotid arteries were measured within 1 cm vessel segment proximal to the carotid bulb in the sagittal planes (Fig. 1). Patients or healthy controls with carotid

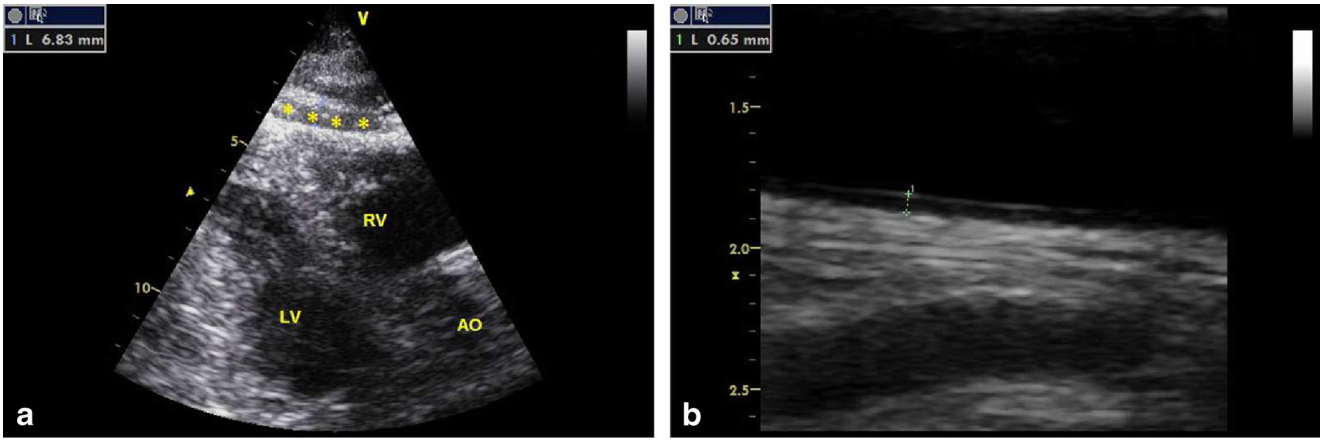

Fig. 1 The demonstration of **a)** epicardial fat tissue (asterisk signs) on parasternal long-axis view of transthoracic echocardiography and **b)** carotid artery intima media thickness measurement on high-resolution B-mode ultrasound

plaques (n=2) detected during CIMT measurement were excluded from the study.

Biochemical and hematological parameters

Peripheral venous blood samples were drawn after 12 h of fasting. Total and differential leukocyte counts were measured with an automated hematologic analyzer (Abbott Cell-Dyn 3700; Abbott Laboratory, Abbott Park, IL). Absolute cell counts were used for all analyses. Total and high-density lipoprotein (HDL) cholesterol, triglycerides, and fasting serum glucose were measured by standard methods. Low-density lipoprotein (LDL) cholesterol was calculated by Friedewald equation.

ADMA assays

Blood samples were immediately centrifuged at 3000 rpm for 10 min, and serums were stored at −80 °C until laboratory testing. The serum ADMA level was determined using an enzyme-linked immunosorbent assay (ELISA) method according to the manufacturer's instructions (DLD Diagnostika GMBH, Hamburg, Germany; reference range, 0.05–2.5 μmol/l). The results were expressed as micromoles/liter.

Statistical analysis

Data were analyzed with SPSS software version 18.0 for Windows (SPSS Inc., Chicago, IL). The Kolmogorov–Smirnov test was used to verify normal distribution of continuous variables. Continuous variables are reported as means±standard deviations, and categorical variables are presented as percentages. The independent sample t test or the Mann–Whitney U test was used for continuous variables, and the Chi-square test was used for categorical variables. The Pearson or Spearman test was used for correlation analyses. Statistical significance was accepted as $p<0.05$. To determine the independent predictors of BD, a multivariate logistic regression analysis was performed with the "Enter" method. A classification table and the Nagelkerke R square were used to evaluate the predictive accuracy of the logistic regression model. Variables with a p value<0.1 in univariate analysis were included in the logistic regression model, and results are presented as odds ratios (OR) with 95 % confidence intervals (CI). A receiver-operating characteristic (ROC) curve analysis was used to determine the optimum cutoff level for the CIMT, serum ADMA level, NLR values, and EFT that best predicted BD.

Results

The mean age of the 36 patients with BD (19 male) was 32±7 years, and that of the 35 healthy control subjects (21 male) was 30±5 years. The baseline demographic, biochemical, and hematological data of the BD patient and control groups are presented in Table 1. Among the 17 patients with active ocular BD, 8 were taking medication (5 cyclosporine [Cyc] plus azathioprine [Aza], 2 Aza, and 1 Cyc), while 5 of 19 patients with inactive ocular BD were on medication (3 Cyc plus Aza, 1 Aza, and 1 Cyc). The mean EFT and NLR values of the patients were significantly higher than those of the controls (4.95±0.79 mm vs. 4.04±0.42 mm; $p<0.001$, and 3.63±1.71 vs. 2.63±1.21; $p=0.007$, respectively). The mean serum ADMA level of the patient group was also significantly higher compared to healthy controls (3.20±0.54 μmol/l vs. 1.96±0.79 μmol/l; $p<0.001$). The other laboratory measurements were similar between the patient and control groups (Table 1). Carotid intima media thickness was thicker in the patient group than in the healthy controls (0.523±0.090 mm vs. 0.417±0.035 mm; $p<0.001$). Disease duration was positively correlated with EFT and CIMT values in BD patients (Table 2, Fig. 2).

Multivariate logistic regression analysis revealed that high serum ADMA level and CIMT values are independently

Table 1 Baseline characteristics of patients with Behçet's disease and control group

Variables	Behçet's disease (n=36)	Controls (n=35)	p value
Age, years	32.1±7.3	30.1±4.9	0.202
BMI, kg/m^2	24.2±2.4	23.4±2.6	0.190
Male gender, n (%)	19 (53 %)	21 (60 %)	0.650
Smoker, n (%)	11 (31 %)	10 (29 %)	0.881
Glucose, mg/dL	92.9±8.2	91.4±8.4	0.464
Urea, mg/dL	27.5±9.3	29.2±5.9	0.386
Creatinine, mg/dL	0.77±0.12	0.79±0.10	0.425
Hemoglobin, g/dL	13.6±1.8	14.4±1.7	0.064
White blood cell, ×10^3/mm^3	9.36±1.87	8.27±1.78	*0.019*
Neutrophil, ×10^3/mm^3	6.55±1.80	5.34±1.56	*0.005*
Lymphocyte, ×10^3/mm^3	2.10±0.78	2.23±0.73	0.482
NLR	3.63±1.71	2.63±1.21	*0.007*
ADMA, μmol/L	3.20±0.54	1.96±0.79	*<0.001*
CIMT, mm	0.523±0.090	0.417±0.035	*<0.001*
EFT, mm	4.95±0.79	4.04±0.42	*<0.001*
LVEF, %	66.9±2.0	67.6±1.9	0.106
LA diameter, mm	33.2±3.0	32.5±3.2	0.327

Data are given as means±SD

ADMA asymmetric dimethylarginine, *BMI* body mass index, *BP* blood pressure, *EFT* epicardial fat thickness, *HDL* high-density lipoprotein, *LA* left atrium, *LDL* low-density lipoprotein, *LVEF* left ventricular ejection fraction, *MPV* mean platelet volume, *NLR* neutrophil to lymphocyte ratio, *SD* standard deviation

associated with the presence of BD. This indicates that every 0.1 mm increase in CIMT and 1.0 μmol/l increase in serum ADMA level were associated with approximately 26-fold and 15-fold increase in the probability of having BD, respectively (Table 3).

In the ROC curve analysis, a serum ADMA level >2.60 μmol/l predicted the presence of BD with 92 % sensitivity and 84 % specificity (ROC area under curve=0.915, 95 % CI 0.842–0.988, p<0.001); and a value of CIMT >0.45 mm predicted the presence of BD with 83 % sensitivity and 84 % specificity (ROC area under curve=0.856, 95 % CI 0.762–0.950, p<0.001; Fig. 3).

Table 2 Correlation analyses (Pearson correlation test) between disease duration and independent variables

Independent variables	Disease duration	
	r coefficient	p value
Age	0.117	0.497
ADMA	0.306	0.069
CIMT	0.501	*0.002*
EFT[a]	0.406	*0.014*
NLR	0.168	0.327

ADMA asymmetric dimethylarginine, *CIMT* carotid intima media thickness, *EFT* epicardial fat thickness, *NLR* neutrophil to lymphocyte ratio

[a] Spearman correlation test

Behçet's disease patients were categorized into two subgroups according to activity of the ocular involvement: active or inactive ocular BD. Neutrophil counts, NLR, and serum ADMA level were significantly higher, and lymphocyte count was significantly lower in the active ocular BD group compared to those in the inactive ocular BD patients (Table 4).

Discussion

Our data demonstrated that CIMT, EFT, serum ADMA level, and NLR were increased in patients with BD compared to healthy subjects. High serum ADMA level and CIMT value were independently associated with the presence of BD. The ROC curve analysis revealed that a serum ADMA level >2.60 μmol/l predicts the presence of BD with 92 % sensitivity and 84 % specificity; and a CIMT value >0.45 mm predicts the presence of BD with 83 % sensitivity and 84 % specificity. Moreover, serum ADMA level and NLR were higher in patients with active ocular involvement compared to those with inactive ocular BD. To the best of our knowledge, this study is the first report showing increased EFT in BD and the association of serum ADMA level and NLR with disease activity of ocular involvement in BD.

Behçet's disease is considered to be a multisystem vasculitis that can affect all types of blood vessels. Endothelial dysfunction, due to vasculitis in BD, plays a crucial role in the pathogenesis of BD's vascular complications. ADMA is an endogenous competitive inhibitor of NO synthase that can modulate NO production [17]. Decreased activity of NO (also known as endothelium-derived relaxing factor) leads to vasoconstriction, monocyte adhesion, and platelet aggregation, which may lead to vascular dysfunction, separately or in combination, seen in BD [18]. Along with increased serum ADMA levels, lower NO levels have been reported to be a significant contributor to ED observed in BD [19–21]. Both the inhibition of NO synthase by ADMA and oxidant/antioxidant imbalance may contribute to decreased bioavailability of NO in BD. High level of serum ADMA has been suggested to be an independent risk factor for ED, coronary heart disease, and cardiovascular mortality [8, 22–24]. Increased serum ADMA level was shown to be independently associated with contrast-induced nephropathy in patients with chronic renal disease who underwent coronary angiography [25]. An epidemiologic study reports that increased serum ADMA level is independently associated with a risk of metabolic syndrome in women [26]. In another study, serum ADMA level was higher in diabetic patients with macrovascular disease (MVD) than those of non-MVD diabetics and healthy controls [27], which indicates that serum ADMA level increases in clinical conditions that are related

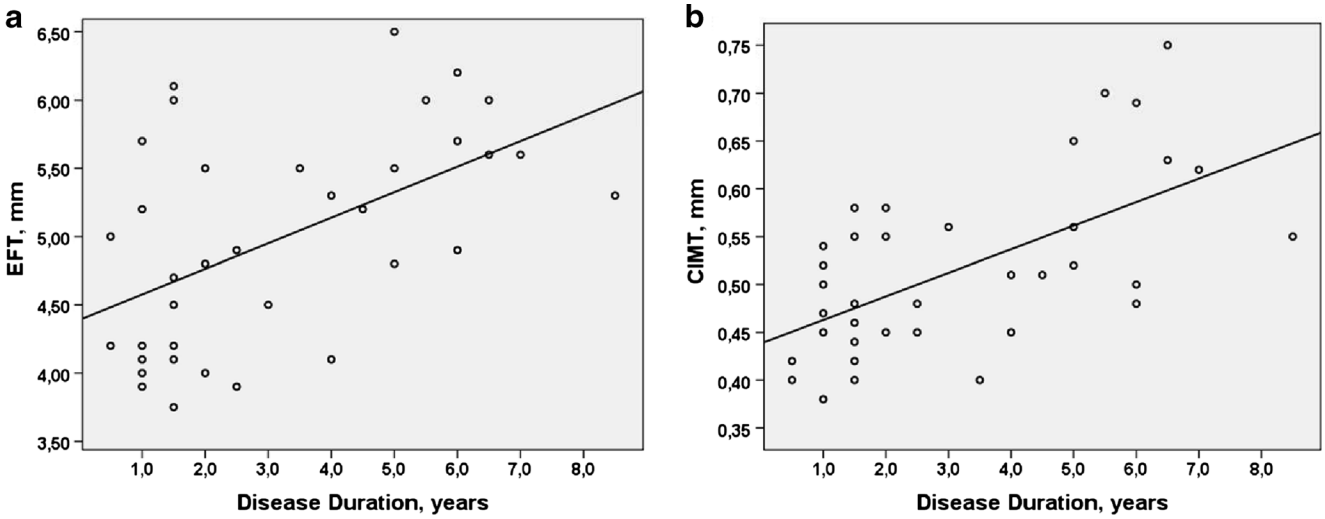

Fig. 2 The scatter plot graphs showing the correlation of disease duration with **a**) epicardial fat thickness (EFT) and **b**) carotid intima media thickness (CIMT)

with ED. A few studies have reported lower serum ADMA concentration and higher NO level in BD suggesting that high NO level may contribute to overall inflammatory process of BD [28, 29]. On the other hand, other studies have reported higher serum ADMA concentration and lower NO level in BD patients compared to healthy controls [21, 30]. A recent study reports that ED, as demonstrated by impaired brachial artery flow-mediated dilatation and elevated serum ADMA level, is a feature of BD [30]. Our results regarding serum ADMA level was consistent with those of previous studies reporting higher ADMA levels in BD patients. In our homogenous study population, serum ADMA level was significantly higher than that of healthy controls. In addition, active ocular BD patients had higher serum ADMA concentration than did inactive ocular BD group, suggesting that serum ADMA level is associated with disease activity of ocular involvement. The etiology of ED in BD is most likely multi-factorial, and high serum ADMA concentration may have contributory effect. Furthermore, together with CIMT, serum ADMA concentration was independently associated with the presence of BD.

In addition to increased serum ADMA level, previous studies have reported evidences of ED in BD patients via demonstrating impaired endothelium-dependent vasodilatation and increased CIMT [31–34]. Increase in CIMT is a sign of structural arterial remodeling which was shown to be related with ED [7]. Previous large-scale prospective studies have reported that increase in CIMT is directly associated with an increased risk of myocardial infarction and stroke [35, 36]. Our results supported the presence of ED in ocular BD patients, as we showed that patients had higher serum ADMA level and CIMT values than did the healthy subjects. The patients with active and inactive ocular BD had similar CIMT values. Nevertheless, CIMT was positively correlated with disease duration, suggesting that thickening of the carotid intima media layer is a result of chronic pathophysiological processes which requires time during the course of BD.

Since BD is a chronic vasculitis, inflammation plays a critical role in pathogenesis. Epicardial fat tissue is a source of some pro-inflammatory and pro-atherogenic cytokines, which mediate immune response. Pro-inflammatory mediators secreted from EFT include interleukin-6, interleukin-1, tumor necrosis factor-α, angiotensinogen, monocyte chemoattractive protein-1, plasminogen activator inhibitor-1, resistin, and free fatty acids [11, 37]. Epicardial fat tissue

Table 3 Multivariate logistic regression analysis (Nagelkerke *R* square of the model is 0.819) to assess predictors of the presence of Behçet's disease

Independent variables	Univariate analysis		Multivariate analysis	
	Odds ratio (95 % CI)	*p*	Odds ratio (95 % CI)	*p*
ADMA	35.97 (5.83–221.75)	<0.001	26.12 (2.39–285.81)	*0.008*
CIMT	19.31 (4.48–83.20)	<0.001	14.95 (1.40–160.00)	*0.025*
EFT	11.46 (3.23–40.67)	<0.001	3.61 (0.40–32.76)	0.253
NLR	1.60 (1.10–2.33)	0.014	1.99 (0.98–4.04)	0.055

ADMA asymmetric dimethylarginine, *CI* confidence interval, *CIMT* carotid artery intima media thickness, *EFT* epicardial fat thickness, *NLR* neutrophil to lymphocyte ratio

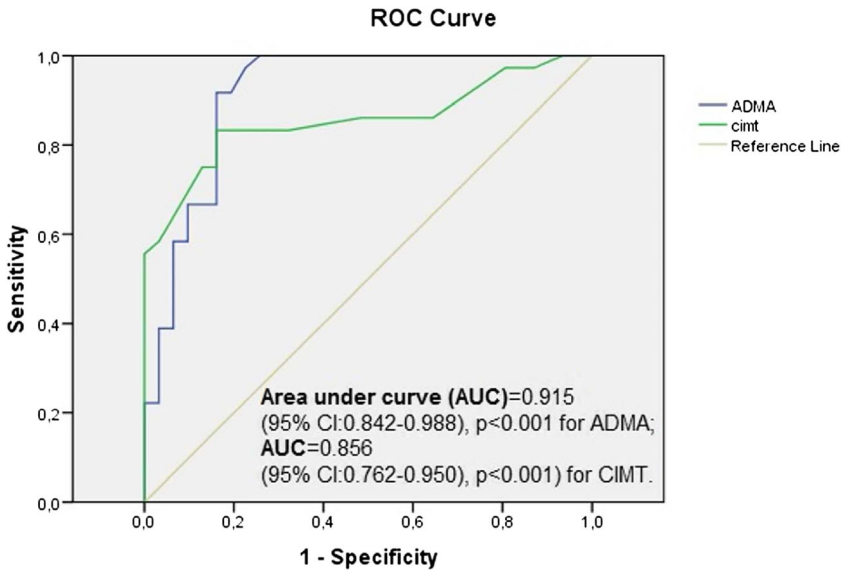

Fig. 3 Receiver operating characteristic (ROC) curve analysis of the serum asymmetric dimethylarginine (ADMA) level and carotid intima media thickness (CIMT) predicting the presence of Behçet's disease

reflects visceral adiposity that has been proposed as a new cardio-metabolic risk factor [16]. Therefore, EFT may play a substantial role in the pathogenesis of various CVDs. Previous studies have shown an association between EFT and ED-associated conditions such as coronary artery disease, pre-eclampsia, arterial stiffness, and hypertension [38–40]. We found that EFT is increased in BD patients compared to healthy controls. To our knowledge, this study is the first report showing increased EFT in BD patients. In addition, disease duration was positively correlated with EFT and CIMT values, which suggest that anatomical changes occur in time during the course of the disease. The patient and control groups had similar body mass indices and they were recruited from the same population during a certain time period, from the age- and gender-matched individuals having the same socio-economic status and none of the participants were athlete or on a training program. The study population did not have any chronic disorders related with EFT other than BD. Thus, EFT, as a part of visceral fat, was expected to be affected by issues only related with BD.

Neutrophil-to-lymphocyte ratio is a novel marker of inflammation that has been studied in various CVDs. A number of pro-inflammatory mediators had been previously shown to be elevated in the serum of BD patients [11, 37], which provide evidence about ongoing inflammatory process during the disease course. Our results support previous studies reporting higher inflammatory markers in BD. We found higher NLR values in patients with BD compared to those of healthy controls. Furthermore, patients with active ocular BD had higher NLR values than did those in the inactive ocular BD group. To our knowledge, this is the first report showing increased NLR values and the association between NLR and disease activity in ocular BD patients.

Table 4 Comparison of laboratory measurements between active and inactive ocular BD patients

Variables	Active ocular BD (n=17)	Inactive ocular BD (n=19)	p
White blood cell, ($\times 10^3$/ml)	9.63±2.35	9.11±1.33	0.406
Neutrophil, ($\times 10^3$/ml)	7.22±1.98	5.95±1.41	*0.032*
Lymphocyte, ($\times 10^3$/ml)	1.77±0.67	2.39±0.77	*0.016*
NLR	4.53±1.67	2.82±1.33	*0.002*
ADMA, (μmol/L)	3.40±0.58	3.03±0.44	*0.036*
CIMT (mm)	0.524±0.101	0.521±0.082	0.921
EFT (mm)	5.01±0.75	4.91±0.85	0.717

Data are given as mean±SD

ADMA asymmetric dimethylarginine, *BD* Behçet's Disease, *CIMT* carotid intima media thickness, *EFT* Epicardial fat thickness, *NLR* neutrophil to lymphocyte ratio, *SD* standard deviation

Study limitations

The study was conducted in a single center and included a relatively small number of patients. The subjects meeting specific criteria were included into this study, and this allowed for the comparison of well-matched groups. However, our results still may not be applicable to the general population. Epicardial fat thickness as measured by transthoracic echocardiography may not reflect the total epicardial fat volume, which differs at various sites around the heart. Echocardiography is an accurate, simple, readily available, radiation-free, and less expensive method than magnetic resonance imaging or computed tomography which are the gold standard diagnostic tools for evaluation of EFT and epicardial fat volume. Despite these limitations, to our knowledge, this is one of the first studies concomitantly evaluating the markers of ED and ongoing inflammation including serum ADMA level, CIMT,

EFT, and NLR in a homogenous group of BD patients with ocular involvement.

Conclusion

In conclusion, we found that CIMT, serum ADMA level, EFT, and NLR were increased in ocular BD patients compared to healthy subjects. In addition, both serum ADMA level and NLR were associated with disease activity of ocular involvement. Increase in disease duration was associated with increase in CIMT and EFT which suggests that anatomical changes occur in time during the disease course. Increased CIMT, serum ADMA level, EFT, and NLR may provide new clues about the role of ED and inflammation in the etiopathogenesis of BD.

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Disclosure None.

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